

(19) World Intellectual Property Organization  
International Bureau



Rec'd PCT/PTO 23 JUN 2005



(43) International Publication Date  
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number  
WO 00/78925 A1

(51) International Patent Classification<sup>7</sup>: C12N 1/20, 1/21, 1/15, 1/19, 15/00

(21) International Application Number: PCT/US00/16806

(22) International Filing Date: 19 June 2000 (19.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/139,889 22 June 1999 (22.06.1999) US

(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US];  
9800 Medical Center Drive, Rockville, MD 20850-3321 (US).

(72) Inventors: BLOOM, Fredric, R.; 19456 Caravan Drive, Germantown, MD 20874 (US). SCHMIDT, Brian, J.; 13503 Crispin Way, Rockville, MD 20853 (US). LIN, Jhy-Jhu; 4 Candlelight Court, Potomac, MD 20854 (US).

(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- With international search report.
- With (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RAPID GROWING MICROORGANISMS FOR BIOTECHNOLOGY APPLICATIONS

(57) Abstract: The present invention provides novel rapidly growing microorganisms and methods for their use in cloning or sub-cloning nucleic acid molecules. The rapid growing microorganisms of the present invention form colonies more rapidly than microorganisms typically used in molecular biology and thus provide a significant improvement in *in vitro* cloning methods used extensively in molecular biology. The invention also relates to kits and compositions used in the methods of the invention.

WO 00/78925 A1

## RAPID GROWING MICROORGANISMS FOR BIOTECHNOLOGY APPLICATIONS

### BACKGROUND OF THE INVENTION

5

#### *Field of the Invention*

The present application relates to the field of biotechnology and, in particular, to the fields of cloning and protein expression.

10 

#### *Related Art*

The fundamental process that sustains the ongoing biotechnology revolution is the cloning of DNA molecules for their further analysis or use. Cloning of DNA molecules has been practiced in the art for many years. A typical cloning protocol will involve identifying a desired DNA molecule, preparing a population of recombinant vectors by ligating the DNA molecule with a vector in a mixture of DNA molecule, vector and an appropriate ligase enzyme, transforming the population of recombinant vectors into a competent microorganism, growing the microorganism for some period of time sufficient to permit the formation of colonies, selecting colonies of microorganisms that potentially contain the desired DNA molecule correctly ligated in the vector, growing a sufficient quantity of each selected colony from which to isolate the recombinant vector, analyzing the isolated vector to ensure that the vector contains the desired DNA molecule and then growing a sufficient quantity of the microorganism that contains the correct recombinant vector to perform whatever subsequent manipulations are required. For details of various cloning procedures the reader may consult Sambrook, *et al.* 1989, Molecular Cloning: A Laboratory Manual 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, specifically incorporated herein by reference.

The typical cloning protocol outlined above thus includes at least three steps that involve growing of a microorganism. Since these growing steps generally require 12-16 hours and are usually performed as overnight incubations, the rate limiting steps for experiments involving cloning of a DNA fragment are the steps requiring growth of a microorganism. Although there are many variations on the basic practice of cloning, virtually all cloning

methods require the insertion of the DNA molecule of interest into a microorganism and growth of the microorganism and, therefore, the speed of virtually every cloning methodology is limited by the rate of growth of the microorganism used for cloning.

5 For most cloning applications, the microorganism of choice is *Escherichia coli* (*E. coli*). Although numerous strains of *E. coli* are known, most cloning applications use one or another derivative of *E. coli* K-12. These derivatives suffer from the slow growth rate discussed above. Other known strains of *E. coli*, such as *E. coli* W (ATCC9637), have a rapid growth rate  
10 when compared to *E. coli* K-12; however, wild type strains of *E. coli* W and other rapid growing strains are not suitable for biotechnology applications for several reasons. First, the genetics of the organism have not been determined to the level of detail required by cloning applications. Thus, those skilled in the art would not know whether the genome of a microorganism contained the  
15 appropriate modifications of a number of genes that would make the microorganism suitable for biotechnology applications. For example, microorganisms are generally *recA*<sup>+</sup> which leads to the formation plasmid multimers and makes the microorganism less suitable for applications that involve the isolation of plasmid. Microorganisms typically contain numerous  
20 protease genes and may degrade overexpressed proteins thereby decreasing the yield of a desired protein product. Microorganisms typically contain a *lac* operon that does not permit alpha complementation and, therefore, the identification of recombinant vectors is more difficult. Further, many microorganisms contain endogenous plasmids that complicate the plasmid  
25 isolation steps necessary for cloning applications. Finally, microorganisms might contain genes coding for nucleases that could cause the degradation of exogenous plasmids.

For a large number of biotechnology applications, a crucial step in the development of the application involves cloning one or more fragments of  
30 DNA. Given the central role of cloning in the development of the biotechnology industry, there has long existed in the art a need for reagents that speed the process of cloning. In particular, there exists a need in the art for microorganisms that have a desirable genotype and a rapid growth rate and

can be employed to speed the cloning process. The present invention meets this long felt need.

### BRIEF SUMMARY OF THE INVENTION

5

The present invention provides microorganisms for biotechnology applications characterized by a rapid growth rate as compared to the microorganisms currently used for these applications. In particular, the present invention provides a rapid growing microorganism that preferably lacks endogenous plasmids and is, therefore, suitable for cloning applications. Because the microorganisms of the present invention form colonies faster than the microorganisms currently in use in cloning applications, the present invention provides an improvement in cloning desired nucleic acid molecules, allowing more rapid identification and isolation of recombinant vectors and clones of interest.

The present invention thus provides a method of cloning that employs a rapid growing microorganism. The method entails constructing a population of recombinant vectors, transforming competent microorganisms capable of rapid growth with the population of recombinant vectors, selecting a transformed microorganism containing one or more recombinant vectors of interest and/or isolating one or more recombinant vectors of interest from the transformed microorganism. In one embodiment, the rapid growing microorganism is of the genus *Escherichia*. In another embodiment, the rapid growing microorganism is an *E. coli*. In a further embodiment, the rapid growing microorganism is an *E. coli* strain W. In a preferred embodiment, the rapid growing microorganism is an *E. coli* strain W lacking endogenous plasmids. In other preferred embodiments, the rapid growing microorganism is selected from a group consisting of BRL3781, BRL3784 and *recA* derivatives thereof. The cloning methods of the present invention may optionally include a step of growing transformed microorganism at an elevated temperature to increase the growth rate of the microorganism, for example, at a temperature greater than 37°C. In a preferred embodiment, the transformed microorganisms may be grown at about 42°C.

The present invention provides a method of producing a protein or peptide which comprises constructing a recombinant vector containing a gene encoding a protein or peptide of interest, transforming the vector into a competent microorganism capable of rapid growth and culturing the transformed microorganism under conditions that cause the transformed microorganism to produce said peptide or protein. In a preferred embodiment, the rapid growing microorganism is of the genus *Escherichia*. In another preferred embodiment, the rapid growing microorganism is an *E. coli*. In another preferred embodiment, the rapid growing microorganism is an *E. coli* strain W. Other embodiments include a microorganism deleted in the *lon* protease. In some preferred embodiments, the microorganism carries a gene encoding a T7 RNA polymerase (RNAP). In other preferred embodiments, the T7 RNAP gene is under the control of a salt inducible promoter. In another preferred embodiment, the rapid growing microorganism does not contain endogenous plasmids.

The present invention also includes a method of producing a microorganism for cloning comprising the steps of obtaining a rapid growing microorganism containing endogenous plasmids and curing the microorganism of endogenous plasmids. In a preferred embodiment, the rapid growing microorganism is of the genus *Escherichia*. In another preferred embodiment, the rapid growing microorganism is an *E. coli*. In another preferred embodiment, the rapid growing microorganism is an *E. coli* strain W. In a related aspect of the present invention, any desired modification or mutation may be made in the microorganisms of the present invention including, but not limited to, alteration of the genotype of the microorganism to a *recA*<sup>-</sup> genotype such as *recA1/recA13* or *recA* deletions, a *lacZ*<sup>-</sup> genotype that allows alpha complementation such as *lacX74 lacZΔM15* or other *lacZ* deletion, a protease deficient genotype such as *Δlon* and/or *ompT*<sup>-</sup>, an endonuclease minus genotype such as *endA1*, a genotype suitable for M13 phage infection by including the F' episome, a restriction negative, modification positive genotype such as *hsdR17*(*r<sub>K</sub>*<sup>-</sup>, *m<sub>K</sub>*<sup>+</sup>), a restriction negative, modification negative genotype such as *hsdS20*(*r<sub>B</sub>*<sup>-</sup>, *m<sub>B</sub>*<sup>-</sup>), a methylase deficient genotype such as *mcrA* and/or *mcrB* and/or *mrr*, a

genotype suitable for taking up large plasmids such as *deoR*, a genotype containing suppressor mutations such as *supE* and/or *supF*. Other suitable modifications are known to those skilled in the art and such modifications are considered to be within the scope of the present invention.

5           The present invention provides a method of transforming a competent microorganism capable of rapid growth including the steps of obtaining a recombinant vector and contacting a competent microorganism of the present invention with the recombinant vector under conditions which cause the rapid growing microorganism to take up the recombinant vector. In a preferred  
10   embodiment, the rapid growing microorganism is of the genus *Escherichia*. In another preferred embodiment, the rapid growing microorganism is an *E. coli*. In another preferred embodiment, the rapid growing microorganism is an *E. coli* strain W. In another preferred embodiment, the rapid growing microorganism is an *E. coli* strain W lacking endogenous vectors. The  
15   methods of the present invention may optionally include the step of growing the transformed microorganism at elevated temperatures to increase the growth rate of the microorganism, for example, at a temperature greater than 37°C. In a preferred embodiment, the transformed microorganisms may be grown at about 42°C.

20           The present invention also includes kits comprising a carrier or receptacle being compartmentalized to receive and hold therein at least one container, wherein the container contains rapid growing microorganisms. The kit optionally further comprises vectors suitable for cloning. In a preferred  
25   embodiment, the kits may contain a vector suitable for recombinational cloning. In a preferred embodiment, the rapid growing microorganisms may be competent. In some preferred embodiments, the rapid growing microorganisms may be chemically competent. In other preferred  
30   embodiments, the rapid growing microorganisms may be electrocompetent. In some preferred embodiments, the kits of the present invention may include enzyme including, but not limited to, restriction enzymes, ligases, and/or polymerases. In other preferred embodiments, the kits of the present invention may include recombination proteins for recombinational cloning. The kits of

the present invention may also comprise instructions or protocols for carrying out the methods of the present invention.

The present invention includes compositions comprising rapid growing microorganisms. In a preferred embodiment, the rapid growing  
5 microorganism may be a competent microorganism. In some preferred embodiments, the rapid growing microorganisms may be chemically competent. In other preferred embodiments, the rapid growing microorganisms may be electrocompetent. The compositions of the present invention may optionally comprise at least one component selected from  
10 buffers or buffering salts, one or more DNA fragments, one or more vectors, one or more recombinant vectors, one or more recombination proteins and one or more ligases. In a preferred embodiment, the compositions of the present invention may comprise a rapid growing microorganism in a glycerol solution. In other preferred embodiments, compositions of the present invention may  
15 comprise rapid growing microorganisms in a buffer. In preferred embodiments, the microorganisms of the present invention may be in a competence buffer. In other preferred embodiments, the compositions of the present invention may comprise a lyophilized rapid growing microorganism.

The present invention includes a method of making competent rapid  
20 growing microorganisms comprising the steps of obtaining a rapid growing microorganism, growing the rapid growing microorganism and treating the rapid growing microorganism to make it competent. In some embodiments of the present invention, treating the microorganisms may include the step of contacting the microorganisms with a solution containing calcium chloride. In  
25 other embodiments, treating may include the step of contacting the microorganisms with water. Embodiments of the invention may include the step of curing the rapid growing microorganism of endogenous plasmids. In a preferred embodiment, the rapid growing microorganism is of the genus *Escherichia*. In another preferred embodiment, the rapid growing  
30 microorganism is an *E. coli*. In another preferred embodiment, the rapid growing microorganism is an *E. coli* strain W.

## BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a restriction map of the 5.5 kb plasmid of ATCC9637.

5

## DETAILED DESCRIPTION OF THE INVENTION

**Definitions**

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and more consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Competent cells or competent microorganisms** as used herein refers to cells or microorganisms having the ability to take up and establish exogenous DNA molecules. Competent cells include, but are not limited to, cells made competent by chemical means, *i.e.* chemically competent cells, as well as cells made competent for electroporation by suspension in a low ionic strength buffer, *i.e.* electrocompetent cells.

**Expression vector** as used herein refers to a vector which is capable of enhancing the expression of a gene or portion of a gene which has been cloned into it, after transformation or transfection into a host cell. The cloned gene is usually placed under the control (*i.e.*, operably linked to) certain control sequences such as promoter sequences. Such promoters include but are not limited to phage lambda  $P_L$  promoter, and the *E. coli lac*, *trp* and *tac* promoters, the T7 promoter and the baculovirus polyhedron promoter. Other suitable promoters will be known to the skilled artisan.

**Gene** as used herein refers to a sequence of nucleotides that is transcribed in a cell. The term includes sequences that code for proteins and/or peptides as well as other sequences that do not code for such proteins or peptides. Examples of genes that do not code for proteins include, but are not limited to, the genes for tRNA, rRNA and the like. A gene includes a promoter sequence to control the transcription of the gene. A gene may also contain other DNA sequence elements that regulate the amount or timing of



transcription. Such sequences elements are seen to include, but are not limited to, enhancers and the like.

Cell or microorganism as used herein, and which terms may be used interchangeably with each other and with the terms "host cell" and "host cell strain," includes microorganisms that can be genetically engineered. Both gram negative and gram positive prokaryotic cells may be used in accordance with the present invention. Typical prokaryotic host cells that may be used in accordance with the present invention include, but are not limited to, microorganisms such as those of the genus *Escherichia* sp. (particularly *E. coli*), *Klebsiella* sp., *Streptomyces* sp., *Streptococcus* sp., *Shigella* sp., *Staphylococcus* sp., *Erwinia* sp., *Klebsiella* sp., *Bacillus* sp. (particularly *B. cereus*, *B. subtilis*, and *B. megaterium*), *Serratia* sp., *Pseudomonas* sp. (particularly *P. aeruginosa* and *P. syringae*) and *Salmonella* sp. (particularly *S. typhi* or *S. typhimurium*). It will be understood, of course, that there are many suitable strains and serotypes of each of the host cell species described herein, any and all of which may be used in accordance with the invention. Preferred as a host cell is *E. coli*, and particularly preferred are *E. coli* strains derived from *E. coli* W.

As used herein, a "derivative" of a specified microorganism is a progeny or other recipient microorganism that contains genetic material obtained directly or indirectly from the specified microorganism. Such a derivative microorganism may, for example, be formed by removing genetic material from a specified microorganism and subsequently introducing it into another microorganism (*i.e.*, the progeny or other recipient microorganism) by any conventional methodology including, but not limited to, transformation, conjugation, electroporation, transduction and the like. A derivative may be formed by introducing one or more mutations into the genome of a microorganism. The mutations may be insertions into the genome of the microorganism. Alternatively, the mutations may be deletions of one or more bases from the genome of the microorganism. In some instances, the mutations may be the alteration of one or more bases in the genome of the microorganism. In addition, one microorganism is a derivative of a parent microorganism if it contains the genome of the parent microorganism but does not contain the same extrachromosomal nucleic acid molecules. Thus, a strain

produced by curing endogenous vectors from a parent strain is a derivative of the parent strain. Examples of mutations or other genetic alterations which may be incorporated into the microorganisms of the present invention include, but are not limited to, mutations or alterations that create: a *recA*<sup>-</sup> genotype such as *recA1/recA13* or *recA* deletions, a *lacZ*<sup>-</sup> genotype that allows alpha complementation such as *lacX74 lacZΔM15* or other *lacZ* deletion, a protease deficient genotype such as *Δlon* and/or *ompT*<sup>-</sup>, an endonuclease minus genotype such as *endA1*, a genotype suitable for M13 phage infection by including the F' episome, a restriction negative, modification positive genotype such as *hsdR17(r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>)*, a restriction negative, modification negative genotype such as *hsdS20(r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>-</sup>)*, a methylase deficient genotype such as *mcrA* and/or *mcrB* and/or *mrr*, a genotype suitable for taking up large plasmids such as *deoR*, a genotype containing suppressor mutations such as *supE* and/or *supF*. Other suitable modifications are known to those skilled in the art and such modifications are considered to be within the scope of the present invention. \*

**Insert or inserts** as used herein refers to one or more desired nucleic acid segments.

**Isolating** as used herein means separating the desired material, component, or composition at least partially from other materials, contaminants, and the like which are not part of the material, component, or composition that has been isolated. For example, "isolating a recombinant vector" means treating a cell, tissue, organ or organism containing the recombinant vector in such a way as to remove at least some of the other nucleic acid molecules (e.g., large nucleic acid molecules) with which it may be associated in the cell, tissue, organ or organism. As one of ordinary skill will appreciate, however, a solution comprising an isolated recombinant vector may comprise one or more buffer salts and/or a solvents, e.g., water or an organic solvent such as acetone, ethanol, methanol, and the like, and yet the nucleic acid molecule may still be considered an "isolated" nucleic acid molecule with respect to its starting materials.

**Plasmid** as used herein refers to a stable extrachromosomal genetic element.

**Promoter** as used herein refers to a DNA sequence that controls the transcription from another DNA sequence. A promoter is generally described as the 5'-region of a gene and is customarily located proximal to the start codon. The transcription of an adjacent DNA segment is initiated at the promoter region. A repressible promoter's rate of transcription decreases in response to a repressing agent. An inducible promoter's rate of transcription increases in response to an inducing agent. A constitutive promoter's rate of transcription is not specifically regulated, though it can vary under the influence of general metabolic conditions.

**Rapid growing microorganism** as used herein refers to a microorganism that grows more rapidly than *E. coli* K-12 derived strains typically used in molecular biology applications. Rapid growing microorganisms produce colonies of a defined size from individual cells faster than non-rapid growing microorganisms. In general, a rapid growing microorganism will have an increased growth rate, such as a growth rate that is greater by 5%, 10%, 25%, 50%, 75%, 100%, 150%, 200% than the growth rate of a reference microorganism. Greater increases in growth rate may be included depending upon the microorganisms compared. A preferred reference strain is a strain such as *E. coli* MM294 (ATCC33625). Other suitable reference strains include DH5 $\alpha$  and DH10B (Life Technologies, Rockville, MD) and any other strain routinely used in cloning applications. The invention also contemplates any microorganism which has an increased growth rate, such as a growth rate that is greater by 5%, 10%, 25%, 50%, 75%, 100%, 150%, 200%, when compared to *E. coli* W more particularly the specified *E. coli* W strains described herein. In the examples set forth below, rapid growing microorganisms of the present invention were identified by a comparison of the time necessary to grow a colony of 1 mm diameter on antibiotic containing LB plates after transformation with a plasmid conferring resistance to the antibiotic.

Rapid growing microorganisms of the present invention may also be identified by a comparison of the doubling time of a putative rapid growing microorganism to the doubling time of a reference strain. The rapid growing microorganisms of the present invention have a faster doubling time than

known strains. Those skilled in the art are capable of determining the doubling time of a microorganism using standard techniques.

In determining whether a microorganism is a rapid growing microorganism, the growth rate is preferably compared to a reference strain having the same or similar genotype. For example, a putative rapid growing microorganism that is *recA*<sup>-</sup> should be compared to a *recA*<sup>-</sup> reference strain. Those skilled in the art will appreciate that a *recA*<sup>-</sup> microorganism may have a slower growth rate than a comparable *recA*<sup>+</sup> microorganism.

**Recombinant microorganism** as used herein refers to any microorganism which contains a desired cloned gene in a recombinant vector, cloning vector or any DNA molecule. The term "recombinant microorganism" is also meant to include those host cells which have been genetically engineered to contain the desired gene on the host chromosome or genome.

**Recombinant vector** as used herein includes any vector containing a fragment of DNA that is not endogenous to the vector.

**Vector** as used herein refers to a nucleic acid molecule (preferably DNA) that provides a useful biological or biochemical property to an Insert. Examples include plasmids, phages, viruses, autonomously replicating sequences (ARS), centromeres, transposons, and other sequences which are able to replicate or be replicated *in vitro* or in a host cell, or to convey a desired nucleic acid segment to a desired location within a host cell. A vector can have one or more restriction endonuclease recognition sites at which the sequences can be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a nucleic acid fragment can be spliced in order to bring about its replication and cloning. Vectors can further provide primer sites, *e.g.*, for PCR, transcriptional and/or translational initiation and/or regulation sites, recombinational signals, replicons, selectable markers, *etc.* Clearly, methods of inserting a desired nucleic acid fragment which do not require the use of homologous recombination, transpositions or restriction enzymes (such as, but not limited to, UDG cloning of PCR fragments (U.S. Patent No. 5,334,575, entirely incorporated herein by reference), T:A cloning, and the like) can also be applied to clone a fragment into a cloning vector to be used according to the present invention. The cloning vector can further contain one or more selectable markers suitable for

use in the identification of cells transformed with the cloning vector.

The present invention may be used with vectors suitable for recombinational cloning as disclosed in United States Patent No. 5, 888,732 which is specifically incorporated herein by reference. Vectors for this purpose may comprise one or more engineered recombination sites. Vectors suitable for recombinational cloning may be linear or circular. When linear, a vector may include DNA segments separated by at least one recombination site. When circular, a vector may include DNA segments separated by at least two recombination sites. In one embodiment, a vector may comprise a first DNA segment and a second DNA segment wherein the first or the second may comprise a selectable marker. In other embodiments, a vector may comprise a first DNA segment and a second DNA segment, the first or the second segment comprising a toxic gene. In other embodiments, a vector may comprise a first DNA segment and a second DNA segment, the first or the second DNA segment comprising an inactive fragment of at least one selectable marker, wherein the fragment of the selectable marker is capable of reconstituting a functional selectable marker when recombined across the first or second recombination site with another inactive fragment of a selectable marker.

In accordance with the invention, any vector may be used. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be used in accordance with the invention. Such vectors may be obtained from, for example, Vector Laboratories Inc., InVitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OriGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. Such vectors may be used for cloning or subcloning nucleic acid molecules of interest and therefore recombinant vectors containing inserts, nucleic acid fragments or genes may also be used in accordance with the invention. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, recombinational cloning transcription vectors, vectors for receiving large inserts (yeast artificial chromosomes (YAC's), bacterial artificial

chromosomes (BAC's) and P1 artificial chromosomes (PAC's)) and the like. Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage  $\lambda$  vectors, baculovirus vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors which have  
5 compatible replicons for use in combination in a single host (e.g., pACYC184 and pBR322) and eukaryotic episomal replication vectors (e.g., pCDM8). The vectors contemplated by the invention include vectors containing inserted or additional nucleic acid fragments or sequences (e.g., recombinant vectors) as well as derivatives or variants of any of the vectors described herein.

10 Expression vectors useful in accordance with the present invention include chromosomal, episomal and virus derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker (such as a tetracycline or ampicillin  
15 resistance genes) and one or more promoters such as the phage lambda  $P_L$  promoter, and/or the *E. coli lac*, *trp* and *tac* promoters, the T7 promoter and the baculovirus polyhedron promoter. Other suitable promoters will be known to the skilled artisan.

Among vectors preferred for use in the present invention include  
20 pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from InVitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV-SPORT1, available from  
25 Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary skill in the applicable arts.

30 The present invention provides novel microorganisms for biotechnology applications characterized by a more rapid growth rate than those microorganisms currently in use in the art. Both gram negative and gram positive prokaryotic cells may be used. The microorganisms of the present

invention may be of any genus of microorganism known to those skilled in the art. The preferred characteristics of the microorganism are a rapid growth rate and the capability to be transformed with and to maintain exogenously applied DNA, in particular, to be transformed with and to maintain recombinant  
5 vectors. In preferred embodiments, host cells that may be used in accordance with the present invention include, but are not limited to, microorganisms such as those of the genera *Escherichia* sp. (particularly *E. coli*), *Klebsiella* sp., *Streptomyces* sp., *Streptococcus* sp., *Shigella* sp., *Staphylococcus* sp., *Erwinia* sp., *Klebsiella* sp., *Bacillus* sp. (particularly *B. cereus*, *B. subtilis*, and  
10 *B. megaterium*), *Serratia* sp., *Pseudomonas* sp. (particularly *P. aeruginosa* and *P. syringae*) and *Salmonella* sp. (particularly *S. typhi* or *S. typhimurium*). In a preferred embodiment, the microorganisms of the present invention are of the genus *Escherichia*. In other preferred embodiments, the microorganisms of the present invention may be of the species *E. coli*. In a preferred  
15 embodiment, the microorganisms of the present invention may be of the *E. coli* strain W. In another preferred embodiment, the present invention includes derivatives of *E. coli* W that do not contain endogenous vectors. In other preferred embodiments, the microorganisms of the present invention may be *E. coli* strains K, B or C.

20 The microorganisms of the present invention may be identified by comparison to known bacterial strains. In general, comparison may be made to one or another derivative of *Escherichia coli* K-12. A well known strain that can serve as a reference strain is *E. coli* MM294 (ATCC33625). Other suitable reference strains include *E. coli* strains specifically describe herein.  
25 Thus, the invention contemplates any microorganism which grow at the same rate or at a faster rate when compared to the *E. coli* W strains of the present invention. Such comparison can be made by any means known to those skilled in the art, including time to colony formation and/or doubling time.

The microorganisms of the present invention preferably form colonies  
30 more rapidly than the strains to which they may be compared. In particular, the microorganisms of the present invention will more rapidly form antibiotic resistant colonies after transformation with a vector containing an antibiotic resistance gene than the microorganisms of the prior art. To identify the microorganisms of the present invention, a potential candidate microorganism

and a reference strain are spread on suitable solid plates, preferably agar media plates known to those skilled in the art, in parallel. The selection and preparation of a suitable solid plate are within the capabilities of those skilled in the art. A suitable plate may prepared using the medium recommended by  
5 the American Type Culture Collection or other suitable media for cultivation of the candidate microorganism. Alternatively, a comparison of the doubling time in liquid media may be used.

The plates may optionally contain an antibiotic if, for example, a competent reference strain is to be compared to a competent, putative rapid  
10 growing microorganism. Both microorganisms can be transformed with a vector that confers an antibiotic resistance to transformants. After transformation, the two microorganisms can be spread onto antibiotic plates in parallel and incubated at an appropriate temperature. The time to the appearance of antibiotic resistant colonies of a specified diameter can be  
15 determined. The rapid growing microorganisms of the present invention will form antibiotic resistant colonies of a specified size more rapidly than the microorganisms presently used for cloning applications. The plates are incubated at the same temperature and the time to colonies of a specified size is determined. In the examples below, a colony size of 1 mm diameter was  
20 used; however, any size may be selected and used. A microorganism that attains the specified size at a faster rate than the reference organism is considered to be a rapid growing organism.

The present invention also comprises a method of cloning employing the rapid growing microorganisms of the present invention. A population of  
25 recombinant vectors containing a desired insert may be constructed using techniques known in the art. For example, DNA of interest may be digested with one or more restriction enzymes to generate a fragment. The fragment may be purified on an agarose gel. A vector is prepared by digestion with the appropriate restriction enzymes. The vector may be further treated with other  
30 enzymes such as alkaline phosphatase or the Klenow fragment of DNA polymerase, and may be gel purified. The DNA fragment is ligated into the vector using an appropriate ligase enzyme to generate a population of recombinant vectors.



Other methods to produce a population of recombinant vectors may be used. For example, a population of recombinant vectors may be produced by recombinational cloning. An insert donor molecule is prepared comprising a DNA of interest flanked by a first and a second recombination site, wherein  
5 the first and the second recombination site do not recombine with each other. The insert donor molecule is contacted with a vector donor molecule comprising a third and a fourth recombination site, wherein the third and the fourth recombination sites do not recombine with each other. The insert donor/vector donor mixture is further contacted with one or more site specific  
10 recombination proteins capable of catalyzing recombination between the first and the third recombination sites and/or the second and the fourth recombination sites thereby allowing recombination to occur and generating a population of recombinant vectors.

Once constructed, the population of recombinant vectors is introduced  
15 into competent, rapid growing microorganisms using any one of the many techniques for the introduction of vector into a microorganism known to those skilled in the art. The transformed microorganisms are grown and recombinant microorganisms, *i.e.* those containing a vector, are selected. In one embodiment, the genotype of the microorganism is suitable for screening  
20 by alpha complementation and the selection step may include the use of a blue/white screen on solid plates containing a chromogenic substrate for  $\beta$ -galactosidase, such as X-gal. The vectors are isolated from the recombinant microorganism and analyzed for the presence of the DNA of interest.

The present invention also comprises a method of producing a desired  
25 protein or peptide utilizing the rapid growing microorganisms of the present invention. The method comprises constructing a recombinant vector containing a gene encoding the desired protein, transforming the vector into a competent, rapid growing microorganism and culturing the transformed microorganism under conditions that cause the transformed microorganism to  
30 produce the desired protein. The recombinant vector may be constructed using the methodology described above. In one embodiment, the recombinant vector will include an inducible promoter to control transcription from the gene coding for the desired protein. In other preferred embodiments, the

genome of the microorganism will contain a gene for the T7 RNA polymerase under the control of an inducible promoter. In other preferred embodiments, the promoter controlling the expression of the T7 RNA polymerase will be inducible by the addition of salt to the growth media. In preferred  
5       embodiments, the rapid growing microorganism is of the genus *Escherichia*. In other preferred embodiments, the rapid growing microorganism is an *E. coli*. In other preferred embodiments, the rapid growing microorganism is an *E. coli* strain W. In another preferred embodiment, the rapid growing microorganism does not contain endogenous plasmid. In other preferred  
10       embodiments, the genotype of the microorganism has been altered to inactivate one or more genes coding for a protease and/or a ribonuclease. In one such preferred embodiment, the rapid growing microorganism does not contain a functional *lon* protease and/or a functional *ompT* protease. In other preferred embodiments, the rapid growing microorganism of the present  
15       invention does not have a functional *maE* gene and/or a functional *mal* gene. In other preferred embodiments the microorganism does not contain functional *lon* protease and/or a functional *ompT* protease and does not contain a functional ribonuclease encoded by the *maE* gene and/or the *mal* gene.

      The present invention also includes kits comprising a carrier or  
20       receptacle being compartmentalized to receive and hold therein at least one container, wherein the container contains rapid growing microorganisms. The kit optionally further comprises vectors suitable for cloning. In a preferred embodiment, the kits may contain a vector suitable for recombinational cloning. Optionally, the kits of the present invention may contain enzymes  
25       useful for cloning. In a preferred embodiment, the kits may contain one or more recombination proteins. In a preferred embodiment, the rapid growing microorganisms may be competent. In some preferred embodiments, the rapid growing microorganisms may be chemically competent. In other preferred embodiments, the rapid growing microorganisms may be electrocompetent.

30       The present invention includes compositions comprising rapid growing microorganisms. In a preferred embodiment, the rapid growing microorganism may be a competent microorganism. In some preferred embodiments, the rapid growing microorganisms may be chemically competent. In other preferred embodiments, the rapid growing microorganisms may be

electrocompetent. The compositions of the present invention may optionally comprise at least one component selected from buffers or buffering salts, one or more DNA fragments, one or more vectors, one or more recombinant vectors, one or more recombination proteins and one or more ligases. In a preferred embodiment, the compositions of the present invention may comprise a rapid growing microorganism in a glycerol solution. In other preferred embodiments, compositions of the present invention may comprise rapid growing microorganisms in a buffer. In preferred embodiments, the microorganisms of the present invention may be in a competence buffer. In other preferred embodiments, the compositions of the present invention may comprise a lyophilized rapid growing microorganism.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

## EXAMPLE 1

### Strain Construction

All strains (listed in table 1) were constructed via bacteriophage P1 mediated transduction (Jeffrey Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, 1972, specifically incorporated herein by reference). *E. coli* strains containing Tn10 insertions suitable for use with the P1 transduction technique can be obtained from the University of Wisconsin. The parental strain for this work was an *E. coli* W strain designated ATCC9637 obtained from the American Type Culture Collection (Manassas, VA). The isolate received was resistant to bacteriophage P1. ATCC9637 was, therefore, converted to a P1 sensitive phenotype by infection with bacteriophage PICmts. PICmts is a bacteriophage P1 derivative which contains a temperature sensitive repressor and contains the chloramphenicol

resistance gene. The bacteriophage forms P1 lysogens at 30°C but replicates lytically at higher temperatures (>37°C). *E. coli* W ATCC9637 was mixed with bacteriophage P1Cmts and chloramphenicol resistant colonies (which are P1Cm lysogens) were selected on LB chloramphenicol plates at 30°C. The chloramphenicol resistant strain was cured of the P1 lysogen by selection for surviving colonies at 42°C. The surviving colonies are now chloramphenicol sensitive. The P1 sensitive derivative of ATCC9637 (BRL3234) was then used for all subsequent work.

10

## EXAMPLE 2

Construction of *E. coli* W *endA*<sup>-</sup>

Competent cells of BRL3234 were prepared by a modification of the method of Hanahan (Doug Hanahan, J. Mol. Biol. 166,557, 1983) as described in United States patent no. 4,981,797 which is specifically incorporated herein by reference. The competent cells were transformed with pCM301 plasmid DNA (Tucker, *et al.*, 1984, Cell 38(1):191-201.), a plasmid which is temperature sensitive for replication. Transformants were selected on ampicillin plates at 30°C. The introduction of the pCM301 plasmid into BRL3234 aided in the identification of *endA*<sup>-</sup> derivatives as described below.

Bacteriophage P1vir was grown on an *E. coli* strain, DB2, which contains an *endA*<sup>-</sup> mutation linked to the *nupG*::Tn10 transposon. The P1 lysate grown on DB3.2 was used to infect BRL3234/pCM301 with selection for tetracycline resistance. The *tet*<sup>r</sup> colonies were then screened for the linked *endA*<sup>-</sup> mutation by determining the ability of the transductants to degrade the pCM301 DNA after preparation of miniprep DNA. Those transductants which degraded the plasmid DNA were *endA*<sup>+</sup> and those which did not degrade pCM301 plasmid DNA were *endA*<sup>-</sup>. The tetracycline resistant, *endA*<sup>-</sup> derivative of BRL3234/pCM301 was designated BRL3573. A derivative of BRL3573 lacking pCM301 was selected by streaking BRL3573 on an LB plate at 42°C and screening colonies for ampicillin sensitivity.

The ampicillin sensitive derivative of BRL3573 was designated BRL3574. The *nupG*::Tn10 transposon was cured from BRL3574 using LB plates containing fusaric acid (Stanley Maloy and William Nunn, J. Bacteriol.

145:1110, 1981). One tetracycline sensitive derivative of BRL3574 was designated BRL3580. BRL3580 is *E. coli* W *endA*<sup>-</sup>.

### EXAMPLE 3

#### 5 Construction of BRL3582 a *recA*<sup>-</sup> *E. coli* W.

A P1Cm lysate was grown on BRL3229. BRL3229 contains a Tn10 transposon linked to a deletion mutation in *recA*. The P1 lysate was used to transduce BRL3580 and tetracycline resistant transductants were selected at 30°C on LB plates containing 20 µg/mL tetracycline. The transductants were re-purified once on LB tetracycline plates and were then screened for sensitivity or resistance to nitrofurantoin on LB plates containing 4 µg/mL nitrofurantoin. *RecA*<sup>+</sup> strains are resistant to nitrofurantoin whereas *recA*<sup>-</sup> strains are sensitive to nitrofurantoin (S Jenkins and P. Bennett J., Bacteriol. 125:1214, 1976). One tetracycline resistant, nitrofurantoin sensitive derivative of BRL3580 was designated BRL3582.

### EXAMPLE 4

#### 20 Isolation of *E. coli* W Derivatives Lacking Native Plasmids

ATCC9637 and all strains derived from ATCC9637 up to and including BRL3580 contain 2 plasmids. The smaller plasmid is approximately 5.5 kb and the larger plasmid is >50 kb. The 5.5 kb plasmid was prepared from ATCC9637 by Lofstrand Labs (Gaithersburg, MD). A restriction map of this plasmid is provided in Figure 1.

25 The restriction map provided cloning sites which could be used to introduce a gene conferring resistance to ampicillin. The ampicillin resistance gene was isolated from plasmid pTrcN2, a pProEX-1 derivative (Life Technologies, Rockville MD). The source of the ampicillin resistance gene is not critical. The following protocol will work with pProEX-1 and may be modified by those skilled in the art depending on the plasmid used as a source of the ampicillin resistance gene. 1 µg of plasmid pTrcN2 was digested with *Bsp*H1 (New England Biolabs) and the ends filled in with Klenow (Life Technologies, Inc). The 1008 bp DNA fragment containing the ampicillin

resistance gene was purified by agarose gel electrophoresis. The 5.5 kb plasmid was digested with SmaI (New England Biolabs) and then treated with TsAP, a temperature sensitive alkaline phosphatase (Life Technologies, Inc.). The DNAs were mixed, treated with T<sub>4</sub> DNA ligase (Life Technologies, Inc.) and transformed into competent ME DH10B cells (Life Technologies, Inc). Ampicillin resistant colonies were selected on LB plates containing 100 µg/mL ampicillin. Several ampicillin resistant colonies were grown in overnight culture and plasmid DNA was prepared and analyzed by electrophoresis on an agarose gel. All ampicillin resistant clones were found to have a plasmid with a molecular weight of 6.5 kb. The DH10B cells containing the plasmid (designated Wamp) were designated BRL3709.

The Wamp plasmid was transformed into competent cells of BRL3580 (*E. coli* W *endA*<sup>-</sup>) with selection for ampicillin resistance. BRL3580, as well as 5 ampicillin resistant transformants, were grown at 37°C in LB broth containing 100 µg/mL ampicillin and the plasmid DNA was isolated and analyzed by agarose gel electrophoresis. The plasmid DNA from BRL3580 had a molecular weight of 5.5 kb whereas the ampicillin resistant transformants had plasmid DNA with a molecular weight of 6.5 kb indicating that the ampicillin resistance gene ~1 kb had been introduced into the 5.5 kb plasmid to give a 6.5 kb plasmid. Further, the 6.5 kb plasmid containing the ampicillin resistance gene had displaced the 5.5 kb plasmid. This is the expected result since both plasmids contained the same origin of replication. The *E. coli* W derivatives containing the 6.5 kb Wamp plasmid were designated BRL3711. Both BRL3580 and BRL3711 also contained the higher molecular weight (>50 kb) plasmid.

## EXAMPLE 5

### Curing BRL3711 of the 6.5 kb Wamp Plasmid

BRL3711 was cured of the Wamp plasmid by growth in LB broth containing SDS. SDS is well known in the literature as a compound which is used to cure plasmids from *E. coli* strains (A. Bharathi and H. Polasa, FEMS Microbiol. Lett, 84:37, 1991, Susana Rosos, Aldo Calzolari, Jose La Torre, Nora Ghittoni, and Cesar Vasquez, J. Bacteriol 155:402, 1983). BRL3711

was grown in LB broth containing 10% SDS at 30°C. After the culture reached the stationary phase, the culture was diluted 1:1000 into fresh LB + 10% SDS for a second cycle. After the second cycle, the survivors were plated on LB plates 30°C and colonies were screened for sensitivity to ampicillin. One isolate, designated BRL3718, was found to be sensitive to ampicillin indicating that the 6.5 kb plasmid had been cured. Miniprep DNA derived from BRL3711 as well as BRL3718 confirmed that BRL3711 had both the smaller and larger plasmids but that BRL3718 had only the larger plasmid.

10

### EXAMPLE 6

#### Preparation of a Derivative of the Large Plasmid Containing an Antibiotic Resistance Gene.

To isolate *E. coli* W derivatives lacking the larger plasmid, antibiotic resistance genes were introduced into the larger plasmid using the Genome Primer System from New England BioLabs. The larger plasmid was isolated from BRL3718 using the standard alkaline-SDS lysis procedure (J. Sambrook, E.F. Fritsch, and T. Maniatis. 1989 Molecular Cloning: A Laboratory Manual 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor NY.). The Genome Priming System was used according to instructions provided by the manufacturer.

Approximately 80 ng of target plasmid DNA was mixed with 20 ng of donor plasmid DNA in a 20 µL reaction. One donor plasmid, pGSP1, donates the gene conferring resistance to kanamycin (Km). The second donor plasmid, pGSP2, donates the gene conferring resistance to chloramphenicol (Cm). The final reactions were diluted 1:10 in water and electroporated into EMax DH10B cells (Life Technologies, Inc.). 20 µL of cells were mixed with 1 µL of the diluted reaction and the cell-DNA combination was electroporated at 420 V, 4000 ohms, 2.4 kV, 16000 kV/cm. 10 µL were expressed in 1 mL SOC for 1 hour 37°C. 100 µL of the expression mix were plated on LB plates containing either 10 µg/mL kanamycin for the pGPS1 reaction or LB plates containing 12.5 µg/mL chloramphenicol for the pGPS2 reaction. 8 transformants from each reaction were analyzed. Plasmid DNA from all 16

colonies had a high molecular weight plasmid which ran on an agarose gel in approximately the same position as the plasmid DNA isolated from BRL3718. In addition, several of the plasmid DNAs were again electroporated into EMax DH10B cells and were shown to confer resistance to either kanamycin or chloramphenicol on the DH10B cells. It was concluded that the genes conferring resistance to either kanamycin or chloramphenicol had been introduced into the large molecular weight plasmid from BRL3718. DH10B cells containing the high molecular weight plasmid which confers resistance to kanamycin have been designated BRL3726. DH10B cells containing the high molecular weight plasmid which confers resistance to chloramphenicol have been designated BRL3727.

#### EXAMPLE 7

##### Construction of Deletion Plasmids.

Plasmid DNA from the strain BRL3726 (DH10B containing the high MW plasmid + Km<sup>r</sup> marker) was prepared. In two separate reactions, 1 µg of plasmid DNA was partially digested with 0.5 and 0.1 units of the restriction enzyme *Sau3A* I (Life Technologies, Inc.) at 37°C for 15 min. The reactions were extracted with phenol/chloroform and precipitated with ethanol. The DNA from each reaction was ligated using T<sub>4</sub> DNA Ligase (Life Technologies, Inc) and transformed into competent ME DH5α cells (Life Technologies, Inc). Colonies were selected on LB plates containing 20 µg/mL kanamycin at 37°C. Chemically competent cells were used because they are not as efficient in taking up high molecular weight plasmid DNA as electrocompetent cells.

The plasmid DNA from 10 kanamycin resistant (from the 0.1 U reaction) colonies was analyzed by agarose gel electrophoresis. The size of the deletion plasmid DNA ranged from ~4.5-15 kb and the plasmids were designated deletion 1 - deletion 10. DH5α cells containing these plasmids were designated BRL3740-1 to BRL3740-10.



## EXAMPLE 8

Curing BRL3718 of the High Molecular Weight Plasmid DNA.

Chemically competent cells of BRL3718 were prepared according to the method of Hanahan (Hanahan D., 1983 J. Mol Biol 166,557) as modified according to United States Patent no. 4,981,797. Chemically competent cells of BRL3718 were transformed with plasmid DNA isolated from BRL3740-1 (deletion 1, ~8 kb) and BRL3740-3 (deletion 3, ~10 kb) and kanamycin resistant colonies were selected on LB plates containing 20 µg/mL kanamycin at 37°C. Four colonies from each transformation were streaked for single-colony isolates onto LB plates containing 20 µg/mL kanamycin at 37°C. Plasmid DNA was isolated from 4, single-colony isolates and analyzed by agarose gel electrophoresis.

The high molecular weight plasmid DNA was readily apparent in miniprep DNA prepared from BRL3718. However, plasmid DNA prepared from the kanamycin resistant transformants did not indicate the presence of the high molecular weight plasmid DNA. Rather, plasmid DNAs with molecular weights characteristic of BRL3740-1 (~8 kb) and BRL3740-3 (~10 kb) were readily visible. It was concluded that the transformation of deletion 1 and deletion 3 plasmid DNA into BRL3718 resulted in replacement of the high molecular weight plasmid DNA (>50 kb) with deletion 1 and deletion 3 DNA. This is the expected result since the high molecular weight plasmid DNA, deletion 1 plasmid DNA and deletion 3 plasmid DNA all share the same origin of replication. The BRL3718 derivatives containing deletion 1 and deletion 3 plasmid DNA were designated BRL3741 and BRL3742, respectively.

## EXAMPLE 9

Curing BRL3741 and 3742 of the Km<sup>r</sup> Plasmids.

BRL3741 and BRL3742 were grown overnight in LB broth containing 10% SDS at 30°C. The cultures were diluted 1:1000 into LB broth containing 10% SDS and incubated again at 30°C. After 2 cycles at 30°C, dilutions of these cultures (1:10<sup>4</sup> and 1:10<sup>6</sup>) were applied to LB plates, incubated at 30°C, and screened for sensitivity to kanamycin. For BRL3741, 15/50 colonies were

sensitive to kanamycin while 9/50 colonies from BRL3742 were sensitive to kanamycin. Plasmid DNA from 2 kanamycin sensitive derivatives of BRL3741 and 2 kanamycin sensitive derivatives of BRL3742 was isolated and analyzed by agarose gel electrophoresis. No plasmid DNA corresponding to the deletion plasmids was observed on the gel. The BRL3741 derivatives cured of the deletion 1 plasmid were designated BRL3756. The BRL3742 derivatives cured of the deletion 3 plasmid were designated BRL3757.

#### EXAMPLE 10

##### 10 Competent Cells of BRL3756 and BRL3757.

Chemically competent cells of BRL3741, BRL3742, BRL3756 and BRL3757 were prepared according to the method of Hanahan (Hanahan D., 1983 J. Mol Biol 166,557) as modified according to United States Patent no. 4,981,797. BRL3741 and BRL3742 were streaked on LB plates containing 20  $\mu$ g/mL kanamycin and the plates were incubated at 28°C for 20 hours. BRL3756(1), BRL3756(2), BRL3757(1) and BRL3757(2) were streaked on LB plates and the plates were incubated 28°C for 20 hours. 5-6 colonies of each strain were picked into 1 mL SOB medium (D. Hanahan J. Mol Biol 166:557 1983). 0.9 mL of the cells were inoculated into 60 mL SOB medium in a 500 mL baffled shake flask. The flasks were placed in an 28°C incubator 250 rpm. When the OD at 550 nm reached 0.25 - 0.33, the cells were harvested. 50 mL of cells of each strain were centrifuged (4°C) and the cells were re-suspended in 4 mL cold CCMB80 buffer (D. Hanahan, J. Jessee and F. Bloom Methods in Enzymology 204:63 1991, specifically incorporated herein by reference). The cells were allowed to sit on ice for 20 min. 220  $\mu$ L were placed in NUNC vials and the cells were frozen in a dry ice ethanol bath. The cells were stored at -70°C.

#### EXAMPLE 11

##### 30 Evaluation of Time to Ampicillin Resistant Colony.

Vials of competent cells (ATCC9637, BRL3718, BRL3741, BRL3742, BRL3756 and BRL3757) were thawed on ice for 20 min. 100  $\mu$ L of cells

were mixed in a cold Falcon 2059 tube with pUC19 (5  $\mu$ L of 10 pg/ $\mu$ L = 50pg). The cells were allowed to sit on ice for 15 min. The cells were heat shocked at 42°C for 45 seconds followed by a 2 minute incubation on ice. 0.9 mL of room temperature SOC was added to each tube and the tubes were shaken at 37°C (250 rpm) for 30 minutes. Aliquots of the expression mix were plated on LB plates containing 100  $\mu$ g/mL ampicillin and the plates were incubated at either 42°C or 37°C. The time to the appearance of 1mm colonies is shown in table 2. At 37°C, ampicillin resistant colonies of 1 mm size required between 7.8 and 8.2 hours and there was no significant difference in time between strains containing both the 5.5 kb plasmid and the >50 kb plasmid (ATCC9637), strains containing only the >50 kb plasmid (BRL3718), strains containing the smaller kanamycin resistant plasmid (BRL3741 and 3742), or strains containing no plasmids (BRL3756 and 3757). In fact at 42°C colonies of 1 mm size required 7.7 hours for all strains tested. It was concluded that the presence or absence of plasmids in *E. coli* W does not significantly affect the time to appearance of colonies after transformation.

## EXAMPLE 12

### 20 Construction of BRL3734.

Electrocompetent cells of BRL3718 were prepared according to a modification of the protocol described in Hanahan *et. al.*, Methods in Enzymology, vol. 204, p. 63 (1991). DNA from BRL3727 isolate 4<sub>6</sub> was used to introduce the plasmid into BRL3718. 20  $\mu$ L of cells were mixed with 1  $\mu$ L of DNA and the cell-DNA mixture was electroporated at 250 V, 2000 ohms, 1.44 kV, 9.6 kV/cm in the Life Technologies Cell-Porator. 10  $\mu$ L were expressed in 1 mL SOC for 60 min 37°C and the expression was plated on LB plates containing 12.5  $\mu$ g/mL chloramphenicol. After 24 hours the colonies were re-purified and analyzed. The miniprep DNA contained a plasmid with a molecular weight approximately the same size as the plasmid found in BRL3718. The *E. coli* W strain containing the chloramphenicol resistant high molecular weight plasmid was designated BRL3734.

## EXAMPLE 13

Curing BRL3734 of the High Molecular Weight Plasmid DNA.

Chemically competent cells of BRL3734 were prepared according to the method of Hanahan (Hanahan D., 1983 J. Mol Biol 166,557) as modified according to United States Patent no. 4,981,797. Chemically competent cells of BRL3734 were transformed with plasmid DNA isolated from BRL3740-1 (deletion 1, ~8 kb) and BRL3740-3 (deletion 3, ~10 kb) and kanamycin resistant colonies were selected on LB plates containing 20 µg/mL kanamycin at 37°C. Four colonies from each transformation were streaked for single-colony isolates onto LB plates containing 20 µg/mL kanamycin at 37°C. Plasmid DNA was isolated from 4, single-colony isolates and analyzed by agarose gel electrophoresis. The high molecular weight plasmid DNA was readily apparent in miniprep DNA prepared from BRL3734. However, plasmid DNA prepared from the kanamycin resistant transformants did not indicate the presence of the high molecular weight plasmid DNA. Rather, plasmid DNA with molecular weight characteristic of BRL3740-1 (~8 kb) and BRL3740-3 (~10 kb) were readily visible. Moreover, BRL3734 containing deletion 1 and deletion 3 plasmids were streaked for single-colony isolates onto LB containing Km 20 µg/mL and LB containing Cm 12.5 µg/mL plates to confirm the presence, or absence, of the desired plasmid DNAs. No growth was observed on the LB + Cm 12.5 µg/mL plates while the formation of single-colony isolates was observed on Km 20 µg/mL plates. It was concluded that the transformation of deletion 1 and deletion 3 plasmid DNA into BRL3734 resulted in replacement of the high molecular weight plasmid DNA (>50 kb) with deletion 1 and deletion 3 DNA. This is the expected result since the high molecular weight plasmid DNA, deletion 1 plasmid DNA and deletion 3 plasmid DNA all share the same origin of replication. The BRL3734 derivatives containing deletion 1 and deletion 3 plasmid DNA were designated BRL3745 and BRL3746, respectively.

30

## EXAMPLE 14

Curing BRL3745 and 3746 of the Km<sup>r</sup> Plasmids.

BRL3745 and BRL3746 were grown overnight in LB broth containing 10% SDS at 30°C. The cultures were diluted 1:1000 into LB broth containing 10% SDS and incubated again at 30°C. After 2 cycles at 30°C, dilutions of these cultures (1:10<sup>6</sup>) were applied to LB plates, incubated at 30°C, and screened for sensitivity to kanamycin. For BRL3745, 22/100 colonies were sensitive to kanamycin while 1/100 colonies from BRL3742 were sensitive to kanamycin. Plasmid DNA from 3 kanamycin sensitive derivatives of BRL3745 and the one kanamycin sensitive derivative of 3746 was isolated and analyzed by agarose gel electrophoresis.

No plasmid DNA corresponding to the deletion 1 and deletion 3 plasmids was observed on the gel after curing. The BRL3745 derivatives cured of the deletion 1 plasmid were designated BRL3762. The BRL3746 derivative cured of the deletion 3 plasmid were designated BRL3763.

## EXAMPLE 15

Competent Cells of BRL3762 and BRL3763.

Chemically competent cells of BRL3745, BRL3746, BRL3762 and BRL3763 were prepared according to the method of Hanahan (Hanahan D., 1983 J. Mol Biol 166,557) as modified according to United States Patent no. 4,981,797. BRL3745 and BRL3746 were streaked on LB plates containing 20 µg/mL kanamycin and the plates were incubated at 28°C for 20 hours. BRL3762(1), BRL3762(2), and BRL3763(1) were streaked on LB plates and the plates were incubated 28°C for 20 hours. 5-6 colonies of each strain were picked into 1 mL SOB medium(D, Hanahan J. Mol Biol 166:557 1983). 0.9 mL of the cells were inoculated into 60 mL SOB medium in a 500 mL baffled shake flask. The flasks were placed in an 28°C incubator 250 rpm. When the OD<sub>550nm</sub> reached 0.25 - 0.33 the cells were harvested. 50 mL of cells of each strain were centrifuged (4°C) and the cells were re-suspended in 4 mL cold CCMB80 buffer (D. Hanahan, J. Jessee and F. Bloom Methods in Enzymology 204:63 1991). The cells were allowed to sit on ice for 20 min.

220 µL were placed in NUNC vials and the cells were frozen in a dry ice ethanol bath. The cells were stored at -70°C.

5

## EXAMPLE 16

Evaluation of Time to Ampicillin Resistant Colony.

One vial of competent cells (ATCC9637, BRL3734, BRL3745, BRL3746, BRL3762 and BRL3763) was thawed on ice for 20 min. 100 µL of cells were mixed in a cold Falcon 2059 tube with pUC19 (5 µL of 10 pg/µL = 50 pg). The cells were allowed to sit on ice for 15 min. The cells were heat shocked at 42°C for 45 seconds followed by a 2 minute incubation on ice. 0.9 mL of room temperature SOC was added to each tube and the tubes were shaken at 37°C (250 rpm) for 30 minutes. Aliquots of the expression mix were plated on LB plates containing 100 µg/mL ampicillin and the plates were incubated at either 42°C or 37°C. The time to the appearance of 1mm colonies is shown in table 3. At 37°C, ampicillin resistant colonies of 1 mm size required 8.0 hours and there was no significant difference in time between strains containing both the 5.5 kb plasmid and the >50 kb plasmid (ATCC9637), strains containing only the >50 kb plasmid (BRL3734), strains containing the smaller kanamycin resistant plasmid (BRL3745 and 3746), or strains containing no plasmids (BRL3762 and 3763). At 42°C, colonies of 1 mm size required 7.3 hours for all strains tested. It was concluded that the presence or absence of plasmids in *E. coli* W does not significantly affect the time to appearance of colonies after transformation. In addition, the data in tables 3 and 4 indicate that incubation of the LB ampicillin plates at 42°C results in the appearance of ampicillin resistant colonies approximately 0.5 hours faster than on plates incubated at 37°C.

30

## EXAMPLE 17

Comparison of Wildtype *E. coli* W and *E. coli* K-12.

Competent cells of *Escherichia coli* strains ATCC9637 (W), BRL3582 (*E. coli* W endA<sup>-</sup> srl::Tn10 recA1398), and ATCC33625 (MM294) were

prepared according to the method of Hanahan (Hanahan D., 1983 J. Mol Biol 166,557) as modified according to United States Patent no. 4,981,797. The competent cells were prepared using CCMB80 buffer (Hanahan,D., Jessee, J., and Bloom, F.R., 1991, Methods in Enzymology 204,63). Max Efficiency  
5 DH5 $\alpha$  competent cells were obtained from Life Technologies Inc.

The competent cells were thawed on ice for 20 minutes. 100  $\mu$ L of the cells were transformed with 50 pg of pUC19 or 50 pg of pBR322 DNA. The cell-DNA mixture was placed on ice for 30 minutes and then heat shocked at 42°C for 45 seconds. The tubes were then placed on ice for 2 minutes. 0.9  
10 mL of SOC (Hanahan 1983) was added to each tube and the tubes were then shaken at 225 rpm for 1 hour at 37°C. Appropriate dilutions were spread on LB plates containing 100  $\mu$ g/mL ampicillin and the plates were incubated at 37°C. The amount of time in hours to the appearance of 1 mm colonies was measured and is shown in Table 4. ATCC9637 yielded colonies in 8 - 8.5  
15 hours compared to approximately 10 hours for ATCC33625, another *recA*<sup>+</sup> strain. *recA*<sup>-</sup> strains were also compared. BRL3582 yielded colonies in approximately 10 hours compared to 16 hours for DH5 $\alpha$ .

20

### EXAMPLE 18

#### Growth of Transformed Microorganisms at an Elevated Temperature.

Using the protocol described in the preceding example, the effects of growth an elevated temperature were analyzed. Incubating the transformed microorganisms on LB ampicillin plates at 42°C resulted in the appearance of  
25 colonies from 0.5 - 1 hour faster compared to plates incubated at 37°C. Plating the cells on plates made from Circle Grow (Bio101) and containing ampicillin at 100  $\mu$ g/mL resulted in the appearance of colonies from 0.5- 1 hour faster compared to the appearance of colonies on LB plates containing ampicillin at 100  $\mu$ g/mL. Thus, the use of elevated temperatures and/or  
30 enriched growth media may facilitate an increased growth rate of the microorganisms of the present invention.

## EXAMPLE 19

Preparation of Derivatives of *E. coli* W Cured of Plasmids.

An isolate of *E. coli* W that has been cured of plasmid, such as BRL3762, BRL3763, BRL3756 or BRL3757, is used to construct derivatives having genotypes desirable for biotechnology applications. Using the P1 transduction technique described above, strains having one or more useful genetic alterations are prepared. Useful genetic alterations include: a *recA*<sup>-</sup> genotype such as *recA1/recA13* or *recA* deletions, a *lacZ*<sup>-</sup> genotype that allows alpha complementation such as *lacX74 lacZΔM15* or other *lacZ* deletion, a protease deficient genotype such as *Δlon* and/or *ompT*<sup>-</sup>; an endonuclease minus genotype such as *endA1*, a genotype suitable for M13 phage infection by including the F' episome, a restriction negative, modification positive genotype such as *hsdR17(r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>)*, a restriction negative, modification negative genotype such as *hsdS20(r<sub>B</sub><sup>-</sup>, m<sub>B</sub><sup>-</sup>)*, a methylase deficient genotype such as *mcrA* and/or *mcrB* and/or *mrr*, a genotype suitable for taking up large plasmids such as *deoR*, a genotype containing suppressor mutations such as *supE* and/or *supF*. Other suitable modifications are known to those skilled in the art and such modifications are considered to be within the scope of the present invention.

In a preferred embodiment, the rapid growing microorganisms of the present invention contains a modified *lac* operon that permits alpha complementation. In order to support alpha complementation, it was necessary to introduce a deletion into the N-terminal region of the genomic β-galactosidase gene. First, a *lacX74* mutation was introduced into BRL3756 and BRL3757 by P1 transduction with a lysate prepared on BRL3759 which contains the *lacX74* mutation linked to a Tn10 insertion. Strains containing the *lacX74* insertion are tetracycline resistant as a result of the Tn10 insertion. Strains were selected on tetracycline containing plates and the resultant strains were designated BRL3760 (derived from BRL3756) and BRL3761 (derived from BRL3757). The strains were cured of the Tn10 insertion by growth in the presence of fusaric acid and the resultant tetracycline sensitive strains containing the *lacX74* mutation were designated BRL3766 and BRL769. These strains were made competent using the modified method of Hanahan as



disclosed above and were then transformed with plasmid containing the alpha fragment of the  $\beta$ -galactosidase gene. The plasmid containing strains were transduced using a lysate prepared on and *E. coli* strains carrying the  $\phi 80dlacZ\Delta M15$  deletion mutation linked to a Tn10 insertion in the *trp* gene.

5 As a result of the insertion in the *trp* gene, strains carrying this mutation require tryptophan in the growth media. Tetracycline resistant strains were selected and were designated BRL3776 (derived from BRL3756 via BRL3760 and BRL3766) and BRL3778 (derived from BRL3757 via BRL3761 and BRL3769). These strains are *lacX74*  $\phi 80dlacZ\Delta M15$  *trp*::Tn10. To restore

10 the wild type *trp* gene, strains BRL3776 and BRL3778 were transduced with a P1 lysate prepared on *E. coli* DH5 $\alpha$  and selected on minimal media minus tryptophan. The strains were spontaneously cured of the alpha fragment containing plasmid and the final alpha complementation strains BRL3781 (from BRL3776) and BRL3784 (from (BRL3778) were isolated. These

15 strains are *lacX74*  $\phi 80dlacZ\Delta M15$ . BRL3781 and BRL3784 were deposited at the Agricultural Research Service Culture Collection (NRRL, 1815 North University Street, Peoria, Illinois, 61064) on June 17, 1999. The deposits were made under the terms of the Budapest Treaty. BRL3781 has been given accession number NRRL No. B-30143 and BRL3784 has been given

20 accession NRRL No. B-30144.

Those skilled in the art will appreciate that other modifications to the genome of the rapid growing microorganisms of the present invention are possible using the techniques described above. *E. coli* containing a desired mutation linked to a Tn10 insertion are readily available from sources well

25 known to those skilled in the art. The desired mutation can be inserted into the genome of a rapid growing microorganism using P1 transduction and then the Tn10 can be cured by growth in the presence of fusaric acid.

In preferred embodiments, the rapid growing microorganisms of the present invention will carry an inducible T7 polymerase gene. In preferred

30 embodiments, the T7 polymerase gene will be under the control of a salt inducible promoter as described by Bhandari, *et al.*, J. Bacteriology, 179(13):4403-4406, 1997 which is specifically incorporated herein by reference. The T7 polymerase gene may be under the control of the salt

inducible promoters of the *proU* locus. Alternatively, the T7 polymerase gene may be under the control of other salt inducible promoters. Other suitable inducible promoters include the *lac* promoter, the *trp* promoter, the *tac* promoter as well as any other inducible promoter known to those skilled in the art. The selection of the appropriate promoters and construction of strains carrying the T7 polymerase under the control of a given promoter are well within the abilities of those of ordinary skill in the art. Optionally, embodiments containing an inducible T7 polymerase gene may contain mutations in one or more protease genes and mutations in one or more ribonuclease genes. Such mutations may be inserted into the genome using the methods described above.

#### EXAMPLE 20

##### 15 Identification of Rapid Growing Microorganisms

Other microorganisms will be screened to identify rapid growing strains. Isolates to be screened are plated on an appropriate solid medium and grown to a defined colony size. The time to reach the defined colony size is compared to the time taken by an *E. coli* K or other strains described herein to reach the same colony size. The microorganisms to be screened include, but are not limited to, microorganisms such as those of the genera *Escherichia* sp. (particularly *E. coli* and, more specifically, *E. coli* strains B, C, W and K)), *Klebsiella* sp., *Streptomyces* sp., *Streptococcus* sp., *Shigella* sp., *Staphylococcus* sp., *Erwinia* sp., *Klebsiella* sp., *Bacillus* sp. (particularly *B. cereus*, *B. subtilis*, and *B. megaterium*), *Serratia* sp., *Pseudomonas* sp. (particularly *P. aeruginosa* and *P. syringae*) and *Salmonella* sp. (particularly *S. typhi* or *S. typhimurium*). A plasmid conferring an antibiotic resistance is transformed into the microorganism to be screened using the techniques described above. The transformed microorganisms are then plated on a solid medium containing antibiotic and then incubated at an appropriate temperature until colonies of a defined size are observed.

## EXAMPLE 21

Cloning Using Rapid Growing Microorganisms.

The rapid growing microorganisms identified above may be used to clone DNA fragments. A population of recombinant vectors comprising a DNA insert having a desired sequence is constructed as described above. The vector may contain a DNA sequence coding for an antibiotic resistance gene and/or may contain one or more marker genes. The population of recombinant vectors is transformed into a rapid growing microorganism rendered competent by any conventional technique. For example, the microorganism is rendered competent by chemical means using the technique of Hanahan discussed above. Alternatively, the microorganism is made competent for electroporation by removing the growth media and placing the microorganism in a medium of low ionic strength. Any method of making the microorganism competent that allows the microorganism to take up exogenously applied DNA and, in particular, recombinant plasmids, is suitable for the practice of the instant invention.

Competent microorganisms are contacted with some or all of the population of recombinant vectors under conditions suitable to cause the uptake of the recombinant vectors into the competent microorganism. Suitable conditions may include a heat shock. For example, the mixture of cells and population of recombinant vectors are heated to 42°C for 45 seconds. Alternatively, suitable conditions may include subjecting a mixture of microorganism and recombinant vector to an electric field.

After the recombinant vector is taken up by the microorganism, the microorganism is grown for a period of time sufficient to allow the expression of an antibiotic resistance gene. After any such period, the microorganism containing the recombinant vector is spread on plates containing the appropriate antibiotic and incubated until colonies are visible. In a preferred embodiment, the plates are incubated from about 4 hours to about 16 hours. In other preferred embodiments, the plates are incubated from about 4 hours to about 8 hours and in other preferred embodiments, the plates are incubated from about 4 hours to about six hours. In a preferred embodiment, the incubation step is performed at a temperature above 37°C at which

temperature the microorganism containing the recombinant plasmid grows more rapidly than it grows at 37°C. In another preferred embodiment, the incubation step is performed at 42°C.

After colonies become visible, some or all of the colonies will be  
5 selected to be grown in liquid culture. The selection process may be by any conventional means. In a preferred embodiment, the microorganism and vector will permit alpha complementation and the selection is by blue/white screening on X-gal plates in the presence of IPTG. In other preferred  
10 embodiments, the selection is by detecting the presence or absence of a marker gene present on the vector. Suitable marker genes include, but are not limited to, the gene coding for luciferase, the gene coding for chloramphenicol acetyl transferase and the gene coding for  $\beta$ -glucuronidase.

The selected colonies are grown in liquid culture for a period of time sufficient to produce a quantity of recombinant microorganisms suitable for  
15 analysis. The recombinant vector will be isolated from the microorganisms. In a preferred embodiment, the period of growth in liquid culture will be from about 2 hours to about 16 hours. In other preferred embodiments, the period of growth in liquid culture will be from about 2 hours to about 8 hours and in other preferred embodiments, the period of growth in liquid culture will be  
20 from about 2 hours to about 4 hours.

The recombinant vector is isolated by any conventional means. In a preferred embodiment, the recombinant vector is isolated by an alkaline lysis "mini-prep" technique. Optionally, the isolation may employ a column purification step. The isolated vector is analyzed by any conventional  
25 technique, for example, by agarose gel electrophoresis of the plasmid with or without prior digestion of the plasmid with one or more restriction enzymes. Other suitable techniques include sequencing of the plasmid. Techniques for determining the DNA sequence of a plasmid are well known to those skilled in the art.

30 Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and

equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

- 5        All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

Table 1: List of strains used in the experiments

<u>Strain</u>	<u>Relevant Genetic Markers</u>	<u>5.5 kb</u>	<u>Plasmids</u>	<u>6.5 kb</u>	<u>&gt;50 kb</u>	<u>other</u>
<i>E. coli</i> W						
ATCC9637		+				
BRL3234	PI sensitive of ATCC9637				+	
BRL3234/pCM301	amp resistant temp sensitive					pCM301
BRL3573	<i>nupG::Tn10 endA'</i>					pCM301
BRL3574	<i>nupG::Tn10 endA'</i>					
BRL3580	<i>endA'</i>					
BRL3582	As 3580 <i>endA srl::Tn10 recA</i> deletion 1398	+			+	
BRL3711	<i>endA-</i>					
BRL3718	<i>endA'</i>		+(Apr)		+	
BRL373410	<i>endA'</i>	-			+(Cm')	
BRL3741	As 3718					deletion 1 (Km')
BRL3742	As 3718	-				deletion 3 (Km')
BRL3756	SDS curing of 3741	-				
BRL3757	SDS curing of 3742	-				
BRL3745	As 3734	-				
BRL3746	As 3734	-				deletion 1 (Km')
BRL3762	SDS curing of 3745	-				deletion 3 (Km')
BRL3763	SDS curing of 3746	-				
BRL3759	DH10B <i>recA' zah281::Tn10 lacX74</i>	-				
BRL3764	DH10B <i>recA' trp::Tn10 <math>\phi</math>80dlacZAM15</i>	-				
BRL3760	<i>lacX74 zah281::Tn10</i>	-				
BRL3761	<i>lacX74 zah281::Tn10</i>	-				
BRL3766	<i>lacX74</i>	-				
BRL3766/pSU39	<i>lacX74</i>	-				pSU39
BRL3769	<i>lacX74</i>	-				
BRL3769/pSU39	<i>lacX74</i>	-				pSU39
BRL3776	<i>lac74 trp::Tn10 <math>\phi</math>80dlacZAM15</i>	-				pSU39
BRL3778	<i>lac74 trp::Tn10 <math>\phi</math>80dlacZAM15</i>	-				pSU39
BRL3781	<i>lac74 trp::Tn10 <math>\phi</math>80dlacZAM15</i>	-				pSU39
BRL3776	<i>lac74 trp::Tn10 <math>\phi</math>80dlacZAM15</i>	-				pSU39

Table 1: List of strains used in the experiments (continued)

<u>Strain</u>	<u>Relevant Genetic Markers</u>	<u>5.5 kb</u>	<u>Plasmids</u> <u>6.5 kb</u>	<u>&gt;50 kb</u>	<u>other</u>
<i>E. coli</i> K-12					
DB3.2	<i>nupG::Tn10 endA<sup>-</sup></i>				
DH10B	<i>endA<sup>-</sup></i>				
BRL3709	As DH10B				
BRL3726	As DH10B		+(Ap <sup>r</sup> )		
BRL3727	As DH10B			+(Km <sup>r</sup> ) +(Cm <sup>r</sup> )	
DH5 $\alpha$					
BRL3740(1)	As DH5 $\alpha$				deletion 1 (Km <sup>r</sup> )
BRL3740(3)	As DH5 $\alpha$				deletion 3 (Km <sup>r</sup> )
<i>E. coli</i> C					
BRL3229	<i>E. coli</i> C srl <sup>+</sup> ::Tn10 <i>recA</i> deletion 1398 PICm <sup>ts</sup>				

**TABLE 2:** Time in hours to ampicillin resistant colonies after transformation with pUC19 DNA.

**Time to 1mm colony size**

5

<u>STRAIN</u>	<u>42°C</u>	<u>37°C</u>
ATCC 9637	7.7	8.2
BRL3718	7.7	7.8
BRL3741	7.7	8.2
BRL3742	7.7	7.8
BRL3756 (1)	7.7	8.2
BRL3756 (2)	7.7	8.2
BRL3757 (1)	7.7	7.8
BRL3757 (2)	7.7	8.2

**TABLE 3:** Time in hours to ampicillin resistant colonies after transformation with pUC19 DNA.

10

**Time to 1mm colony size**

<u>STRAIN</u>	<u>42°C</u>	<u>37°C</u>
ATCC 9637	7.3	8.0
BRL3734	7.3	8.0
BRL3745	7.3	8.0
BRL3746	7.3	8.0
BRL3762 (1)	7.3	8.0
BRL3762(2)	7.3	8.0
BRL3763	7.3	8.0



**Table 4:** Time in hours to ampicillin resistant colonies after transformation with pUC19 DNA

Time to 1mm colony size			
5	<u>STRAIN</u>	<u>pUC19</u>	<u>pBR322</u>
	ATCC 9637 (W) <i>recA</i> <sup>+</sup>	8.0	8.5
10	BRL3582(6) W <i>recA</i> <sup>-</sup>	10.25	ND
	MM294 <i>recA</i> <sup>+</sup>	10.25	10.25
	DH5α <i>recA</i> <sup>-</sup>	16.0	16.0
15			

40.1

Applicant's or agent's file reference number 0942.497PC01	International application No. Tc : assigned : 16806
--	---

**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**  
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page __32__, line __19__.	
<b>B. IDENTIFICATION OF DEPOSIT</b>	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution Agricultural Research Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 N. University Street Peoria, Illinois 61604 United States of America	
Date of deposit June 17, 1999	Accession Number NRRL B-30143
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
<i>Escherichia coli</i> BRL 3781 endA	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer <i>Virginia L. Lely</i>	Authorized officer

40.2

Applicant's or agent's file  
reference number 0942.497PC01

International application No. T. Je assigned.

**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**  
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 32, line 20.**B. IDENTIFICATION OF DEPOSIT**Further deposits are identified on an additional sheet ☐Name of depositary institution  
Agricultural Research Culture Collection (NRRL)Address of depositary institution (including postal code and country)  
1815 N. University Street  
Peoria, Illinois 61604  
United States of America

Date of deposit June 17, 1999

Accession Number NRRL B-30144

**C. ADDITIONAL INDICATIONS** (leave blank if not applicable)This information is continued on an additional sheet ☐*Escherichia coli* BRL 3784 endA**D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)**E. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

*Virginia L. Lely*

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

## WHAT IS CLAIMED IS:

1. A rapid growing microorganism lacking endogenous plasmids.
- 5 2. The microorganism according to claim 1, wherein the rapid growing microorganism is of the genus *Escherichia*.
3. The microorganism according to claim 2, wherein the rapid growing microorganism is an *E. coli*.
- 10 4. The microorganism according to claim 3, wherein the rapid growing microorganism is an *E. coli* strain W.
5. The microorganism according to claim 4, wherein the rapid growing  
15 microorganism is selected from a group consisting of BRL3781 BRL3784 and *recA*<sup>-</sup> derivatives thereof.
6. A method of cloning, comprising the steps of:  
constructing a population of recombinant vectors;  
20 transforming a competent microorganism capable of rapid growth with the recombinant vector; and  
selecting the transformed microorganism containing the recombinant vector.
- 25 7. The method according to claim 6, wherein the rapid growing microorganism is of the genus *Escherichia*.
8. The method according to claim 7, wherein the rapid growing microorganism is and *E. coli*.
- 30 9. The method according to claim 8, wherein the rapid growing microorganism is an *E. coli* strain W.
10. The method according to claim 9, wherein the rapid growing microorganism does not contain endogenous vectors.

11. The method according to claim 6, further comprising the step of isolating said recombinant vector from said transformed microorganism.
- 5 12. The method according to claim 7, further comprising the step of growing the transformed microorganism at a temperature greater than 37°C.
13. The method according to claim 12, wherein the temperature is about 42°C.
- 10 14. The method according to claim 6, further comprising the step of growing the transformed microorganism at about 42°C.
- 15 15. A method of producing a protein or peptide, comprising the steps of :  
constructing a recombinant vector containing a gene encoding a  
protein or peptide protein;  
transforming the vector into a competent microorganism capable of  
rapid growth; and  
culturing the transformed microorganism under conditions that cause  
20 the transformed microorganism to produce said protein or peptide.
16. The method according to claim 15, wherein the rapid growing microorganism is of the genus *Escherichia*.
- 25 17. The method according to claim 16, wherein the rapid growing microorganism is an *E. coli*.
18. The method according to claim 17, wherein the rapid growing microorganism is an *E. coli* strain W.
- 30 19. The method according to claim 18, wherein the rapid growing microorganism does not contain endogenous plasmids.

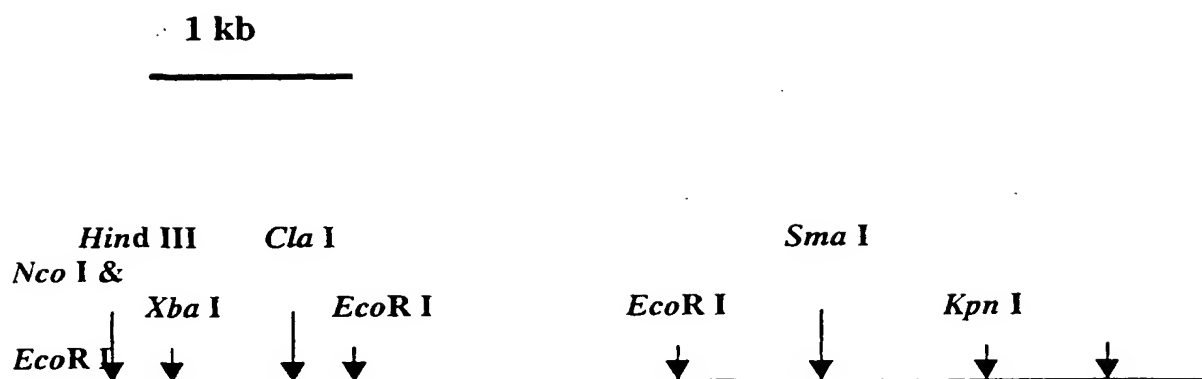
20. A method of producing a microorganism for cloning, comprising the steps of:
- obtaining a rapidly growing microorganism having endogenous plasmids; and
- 5 curing the microorganism of endogenous plasmids.
21. The method according to claim 20, wherein the microorganism is an *E. coli* W.
- 10 22. A method of transforming a rapid growing microorganism, comprising the steps of :
- obtaining a competent microorganism capable of rapid growth; and
- incubating said competent microorganism in the presence of one or more vectors under conditions which cause said one or more vectors to be
- 15 taken up by the microorganism.
23. The method according to claim 22, wherein the rapid growing microorganism is of the genus *Escherichia*.
- 20 24. The method according to claim 23, wherein the rapid growing microorganism is an *E. coli*.
25. The method according to claim 24, wherein the rapid growing microorganism is an *E. coli* strain W.
- 25 26. The method according to claim 25, wherein the rapid growing microorganism does not contain endogenous plasmids.
27. A kit for cloning comprising a container containing a rapid growing
- 30 microorganism.
28. The kit according to claim 27, further comprising one or more vectors.

29. The kit according to claim 28, wherein said kit further comprises at least one component selected from one or more restriction enzymes, one or more ligase enzymes and one or more polymerases.
- 5 30. The kit according to claim 29, further comprising a container containing a recombination protein.
31. The kit according to claim 27, wherein the rapid growing microorganism is competent.
- 10 32. The kit according to claim 31, wherein the rapid growing microorganism is chemically competent.
33. The kit according to claim 31, wherein the rapid growing microorganism is electrocompetent.
- 15 34. A composition comprising rapid growing microorganisms.
35. The composition of claim 34, further comprising a component selected from a group consisting of a glycerol solution and a competence buffer.
- 20 36. The composition of claim 34, further comprising at least one component selected from one or more DNA fragments, one or more ligase enzymes, one or more vectors, one or more buffering salts and one or more recombination proteins.
- 25 37. A method of making competent rapid growing microorganisms, comprising the steps of:  
obtaining a rapid growing microorganism; and  
30 treating the rapid growing microorganism to make it competent.
38. The method of claim 37, further comprising the step of curing the rapid growing microorganism of endogenous vectors.

39. The method according to claim 37, wherein the rapid growing microorganism is of the genus *Escherichia*.
40. The method according to claim 39, wherein the rapid growing  
5 microorganism is an *E. coli*.
41. The method according to claim 40, wherein the rapid growing microorganism is an *E. coli* strain W.



## FIGURE 1



**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**  
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>32</u> , line <u>19</u> .	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
Name of depositary institution Agricultural Research Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 N. University Street Peoria, Illinois 61604 United States of America	
Date of deposit     June 17, 1999	Accession Number NRRL B-30143
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<b><i>Escherichia coli</i> BRL 3781 endA</b>	
In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

<p style="text-align: center;">For receiving Office use only</p> <div style="border: 1px solid black; padding: 5px; margin-bottom: 5px;"> <input checked="" type="checkbox"/> This sheet was received with the international application         </div> <div style="border: 1px solid black; padding: 5px;">           Authorized officer  </div>	<p style="text-align: center;">For International Bureau use only</p> <div style="border: 1px solid black; padding: 5px; margin-bottom: 5px;"> <input type="checkbox"/> This sheet was received by the International Bureau on:         </div> <div style="border: 1px solid black; padding: 5px; height: 40px;">           Authorized officer         </div>
---	--

*Escherichia coli* BRL 3781 endA

Page 2 of 3

**AUSTRALIA**

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

**CANADA**

The applicant hereby requests that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

**DENMARK**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

**FINLAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the National Board of Patents and Registration or any person approved by the applicant in the individual case.

**ICELAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Icelandic Patent Office), or has been finally decided upon by the Icelandic Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

*Escherichia coli* BRL 3781 endA

Page 3 of 3

**NETHERLANDS**

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

**NORWAY**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Norwegian Patent office or any person approved by the applicant in the individual case.

**SINGAPORE**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

**SWEDEN**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

**UNITED KINGDOM**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**  
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>32</u> , line <u>20</u> .	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Agricultural Research Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 N. University Street Peoria, Illinois 61604 United States of America	
Date of deposit      June 17, 1999	Accession Number NRRL B-30144
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<b><i>Escherichia coli</i> BRL 3784 endA</b>	
In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")	

<p style="text-align: center;">For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer <i>Virginia L. Lily</i></p>	<p style="text-align: center;">For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>
--	--

*Escherichia coli* BRL 3784 endA

Page 2 of 3

**AUSTRALIA**

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

**CANADA**

The applicant hereby requests that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

**DENMARK**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

**FINLAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the National Board of Patents and Registration or any person approved by the applicant in the individual case.

**ICELAND**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Icelandic Patent Office), or has been finally decided upon by the Icelandic Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

*Escherichia coli* BRL 3784 endA

Page 3 of 3

**NETHERLANDS**

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

**NORWAY**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Norwegian Patent office or any person approved by the applicant in the individual case.

**SINGAPORE**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

**SWEDEN**

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

**UNITED KINGDOM**

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/16806

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 1/20, 1/21, 1/15, 1/19, 15/00

US CL : 435/252.1, 69.1, 254.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.1, 69.1, 254.1


Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CAPLUS, EMBASE, WEST, BIOTECHNO, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P — Y, P	US 6,071,733 A (MURAMATSU et al) 06 June 2000 (06.06.2000), see entire document.	1-3, 6-8, 11, 12, 15-17, 22-24, 34 ----- 13, 14, 27, 28-33, 35-39
X — Y	ROSAS et al. Involvement of a Plasmid in <i>Escherichia coli</i> Envelope Alterations. Journal of Bacteriology. July 1983, Vol. 155, No. 1, pages 402-406, see entire document.	1-3, 34 — 4-33, 35-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 24 AUGUST 2000	Date of mailing of the international search report 06 SEP 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SEAN MCGARRY  Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)\*